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A short synthetic peptide inhibits signal transduction, migration and angiogenesis mediated by Tie2 receptor

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Tie2, an endothelial cell-specific receptor kinase, has an important role in tumour angiogenesis. In an attempt to identify peptides that specifically interact with and block the Tie2 pathway, a phage-displayed peptide library was screened on a recombinant Tie2 receptor. One peptide, NLLMAAS, completely abolished the binding to Tie2 of both angiopoietin 2 and angiopoietin 1 (Ang1). We further show that NLLMAAS specifically suppresses both Ang1-induced ERK activity and migration in human umbilical endothelial cells. Moreover, *in vivo*, this peptide inhibits angiogenesis in the chick chorioallantoic membrane assay. NLLMAAS is the first peptide described to interact with Tie2. Our results demonstrate that it is an efficient and specific antagonist of the binding of Tie2 ligands, and suggest that this peptide or its derivates may have potential applications in the treatment of angiogenesis diseases. It also represents a potent tool to dissect the molecular mechanisms involved in the Tie2 pathway.

Keywords: angiogenesis; Tie2; antagonist peptides; phage display; angiopoietin; ERK signalling

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INTRODUCTION

Angiogenesis, the formation of new blood vessels sprouting from pre-existing vasculature, is required for embryonic development, several female reproductive functions, and wound healing and other repair processes. Angiogenesis also occurs in several diseases, and its importance in solid tumour growth and metastasis has been demonstrated in multiple studies (reviewed in Carmeliet, 2003). The generation of new capillaries and their remodelling involve a multistep process. This process includes the destabilization of established vessels, endothelial cell migration and proliferation, and the formation of new vascular tubes, which are stabilized by mesenchymal components. Blocking any one of these steps would inhibit the formation of new vessels, and therefore affect tumour growth and generation of metastases. Indeed, fibroblast growth factor 2 (FGF2), an angiogenic factor, has profound effects on endothelial cells, and also on many other cell types on which its receptors are expressed. Until recently, vascular endothelial growth factor (VEGF) was the only growth factor proved to be specific and critical for blood vessel formation.

The angiopoietins/Tie2 receptor pathway has been recently identified as another endothelial-cell-specific proangiogenic system, which has a critical role in promoting vascular homeostasis and vessel maturation, as well as vascular destabilization and remodelling (Sato *et al*, 1995). The Tie2 tyrosine kinase receptor is expressed specifically on endothelial cells (Dumont *et al*, 1992; Suri *et al*, 1996). The interruption of Tie2 signalling with soluble, dominant-negative receptors can significantly inhibit tumour growth in mice (Lin *et al*, 1997; Siemeister *et al*, 1999), therefore indicating that Tie2 antagonists could be promising candidates for the treatment of tumour-induced angiogenesis. For instance, an angiopoietin 1 (Ang1)-antisense RNA approach reduces tumour growth and tumour angiogenesis (Shim *et al*, 2001). Recently, a nuclease-resistant RNA aptamer that binds angiopoietin 2 (Ang2) has been reported to inhibit both Ang2 signalling and FGF2 angiogenesis (White *et al*, 2003).

Ang1 acts as an agonist of Tie2 (Davis *et al*, 1996), whereas Ang2 appears to be a context-dependent antagonist/agonist (Maisonpierre *et al*, 1997; Mochizuki *et al*, 2002). Targeted disruption of Ang1- and Tie2-coding genes and overexpression of Ang2 result in embryonic death with similar vascular defects (Dumont *et al*, 1994; Sato *et al*, 1995; Suri *et al*, 1996; Maisonpierre *et al*, 1997). Ang1 is involved in normal interactions between endothelial cells and their underlying supporting pericytes, as well as in the maintenance of vascular stability. *In vitro* studies have demonstrated that Ang1 induces endothelial cell migration (Witzenbichler *et al*, 1998), sprouting and formation of tubule-like vascular structures (Papapetropoulos *et al*, 1999). Furthermore, it protects endothelial cells from apoptosis (Papapetropoulos *et al*, 2000). The recent Ang2 gene knockout

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mouse model has demonstrated that Ang2 is required for subsequent angiogenic remodelling and seems to act as a Tie2 agonist in the lymphatic system (Gale *et al*, 2002).

The screening of phage-displayed libraries is a powerful technique for identifying peptides that mimic protein surfaces and exhibit selectivity for their targets (Smith, 1985). Several agonists or antagonists of cell membrane receptors have been successfully identified using this method (Cwirla *et al*, 1997; Binétruy-Tournaire *et al*, 2000). In this study, we attempted to identify peptides that would be able to block the binding of angiopoietins to Tie2. A random 7-mer peptide library displayed on the surface of filamentous bacteriophages was screened by biopanning against the extracellular domain of Tie2. This led to the isolation of peptide T4-(NLLMAAS), which not only competed with Ang1/Ang2 binding to Tie2 but also specifically inhibited Ang1-induced signal transduction and migration in human endothelial cells *in vitro*. A local delivery of this peptide inhibited angiogenesis *in vivo* in the chick chorioallantoic membrane (CAM) assay. Thus, NLLMAAS, or its derivates, could constitute a good lead for the development of anti-angiogenic drugs against cancer and angiogenic diseases, and it should also be a useful tool to clarify the mechanisms of angiopoietin/Tie2 signalling.

RESULTS AND DISCUSSION

To select peptides binding Tie2, a random 7-mer library was screened for binding to Tie2-Fc. At the end of the selection, 48 clones were isolated and sequenced, showing that 11 different peptides were represented (T1–T11). Each selected clone was assayed by ELISA for binding to Tie2-Fc. To quantify only the binding to Tie2, the signal measured on an Fc-coated surface was subtracted. Nondisplaying M13 phage particles were used as negative controls (Fig 1). All the tested clones gave a significant ELISA signal, demonstrating specific binding to Tie2. Clones T4, T6, T7 and T8, which gave the highest signal, were chosen for further experiments. Table 1 shows that only T7 and T8 share a sequence homology, with two identical residues: RH.

Six synthetic peptides, T1, T4, T6, T7, T8 and T10 (T1 and T10 as negative controls), were then produced and their ability to compete with Ang2 for binding to Tie2 was tested by ELISA, using a fixed peptide concentration (500 μM). We had verified beforehand that recombinant Ang2 and Ang1 specifically bound to Tie2-Fc but not to TrkB-Fc, Tie1-Fc or Fc (data not shown). The results show that only peptide T4 inhibited Ang2 binding (Fig 2A). The other peptides did not inhibit Ang2 or Ang1 binding to Tie2 even when the peptide concentration was increased to 2 mM (data not shown). T4 abolished Ang2 and Ang1 binding to Tie2 in a dose-dependent manner (Fig 2B). The other selected peptides, which are poor competitors, might bind Tie2 in regions distant from the Ang1/Ang2 binding site. Another explanation may be that the

Table 1 | Multiple alignment of selected clones

Consensus motif	No consensus motif
T7 HHHRHSF	T4 NLLMAAS
T8 HPWLTRH	T6 KLWVIPK

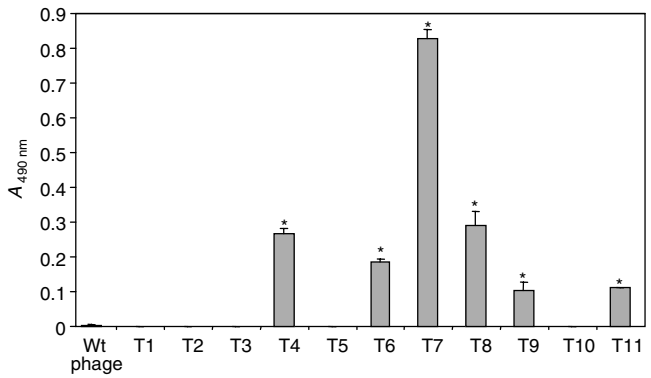

Fig 1 | Selected phage-displayed peptides specifically bind to Tie2. The binding of clones was analysed by ELISA as described in the Materials and methods section, and was compared to that of wild-type M13 phage particles as control (Wt phage). Results are representative of three independent assays. *$P<0.05$ versus control.

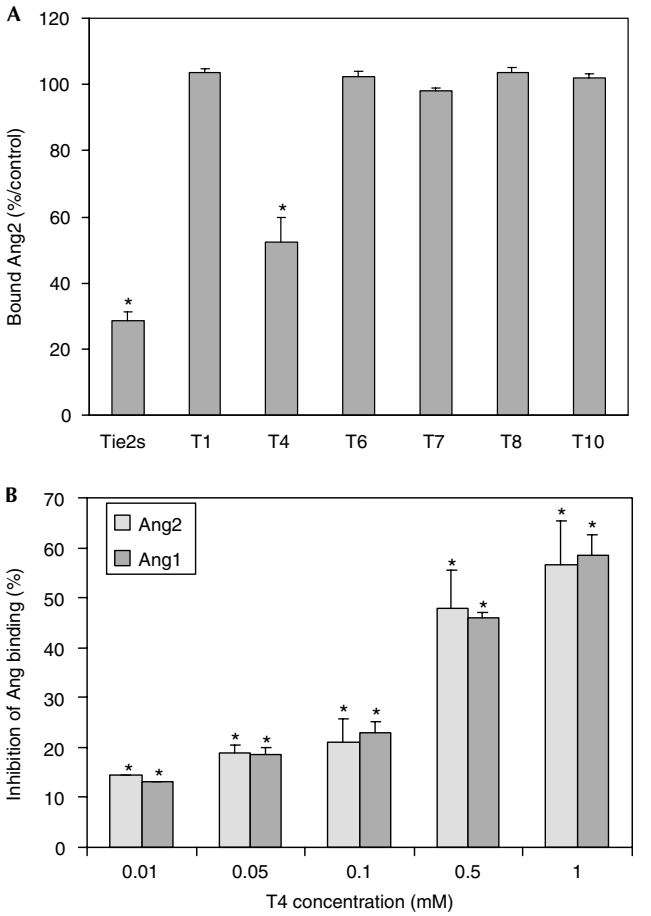

Fig 2 | A synthetic peptide competes with Ang2 for Tie2 binding in ELISA. (**A**) Peptides were tested in competition with Ang2 for binding to Tie2-Fc. As a positive control, Tie2s was tested under the same conditions. (**B**) Increasing concentrations of peptide T4 were tested in competition with Ang2 or Ang1 for binding to Tie2. Data represent the mean and standard deviation of triplicates. Similar results were obtained in three independent experiments. *$P<0.05$ versus control.

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phage peptide has a higher avidity than the free peptide. Indeed, each phage carries several copies of the minor coat protein pIII-fused heptapeptide, and this may result in a multivalent binding of the phage to Tie2-coated receptors. Finally, our observation might also be due to the fact that, unlike synthetic peptides, phage-linked peptides can be conformationally constrained by the phage coat proteins.

On the basis of the ELISA competition assay, peptide T4 was chosen for further studies. The inhibition by peptide T4 of Ang1/Ang2 binding to Tie2 was further characterized by surface plasmon resonance (Fig 3). The K_i values were $3.2(\pm 0.3)\times 10^{-4}$ and $3.2(\pm 0.8)\times 10^{-4}$ M, respectively. On the contrary, T7 was unable to compete with Ang2 or Ang1 for binding to Tie2 (Fig 3). The K_i values of our peptide could be improved by chemical modifications or site-directed mutagenesis. For example, the affinity of P-selectin-binding peptide was increased almost 800-fold through the introduction of a gallic acid moiety at the N-terminus (Appeldoorn *et al*, 2003).

As Tie2 is an endothelial-cell-restricted receptor, we tested whether T4 could inhibit Ang1 signal transduction in human umbilical vein endothelial cells (HUVECs). Ang1 had been previously shown to activate the MAPK signalling cascade in HUVECs. The pharmacological inhibition of ERK activation with PD98059 suppressed Ang1-induced migration (Kim *et al*, 2002) as well as the anti-apoptotic properties of Ang1 (Harfouche *et al*, 2003). We first measured the phosphorylation of ERK1 and ERK2 in response to Ang1 in HUVECs. Figure 4A shows that Ang1 induced a strong p42/p44 phosphorylation. Ang1 binding to Tie2 expressed by endothelial cells was specifically responsible for this MAPK activation, as it was completely abolished by recombinant soluble Tie2 receptor. To determine the activity of peptide T4, HUVECs were stimulated with Ang1 in the presence or absence of a series of concentrations of T4 (Fig 4B). The results show that T4 completely inhibited the activation of MAPK induced by Ang1, whereas peptide T7 at the same concentration had no inhibitory effect. T4 suppressed Ang1-induced MAPK activity in a dose-dependent manner with an IC_{50} of 150 or 200 μM. The peptide alone did not activate MAPK phosphorylation. We then explored the specificity of peptide T4 by evaluating its action on another receptor tyrosine kinase, FGF-R, which is expressed in HUVECs. Although T4 inhibits the ERK activation by Ang1, it had no effect on the MAPK activation in response to the FGF-R agonist FGF2 (Fig 4C).

In vitro experiments have shown that Ang1 has little effect on proliferation, but that it potently stimulates endothelial cell

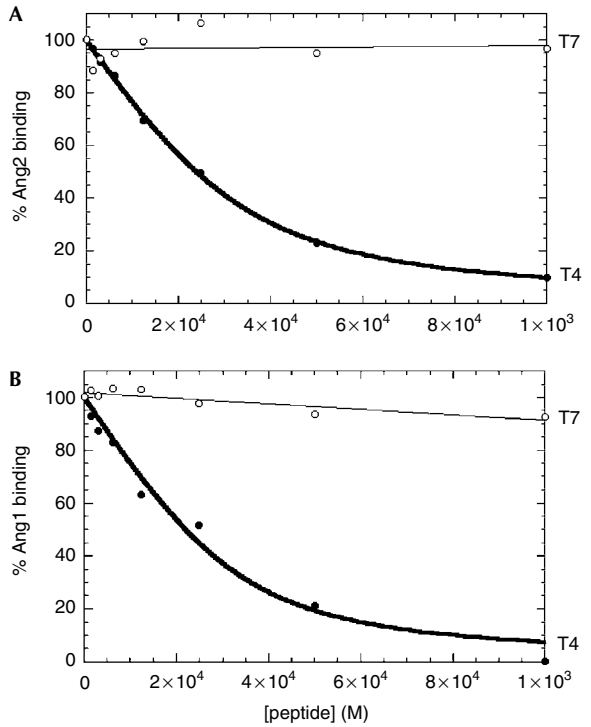

Fig 3 | T4 inhibits the binding of Ang1 and Ang2 to Tie2 in a dose-dependent manner in Biacore experiments. Ang1 or Ang2 were injected onto a Tie2 surface in the presence of a range of concentrations of peptides T4 or T7 (0–1 mM). The association rate was measured for each peptide concentration and divided by that obtained in the absence of peptide: the ratios (in %) are plotted for Ang2 (Fig 3A) and Ang1 (Fig 3B) against the concentration of T4 (bold lines) or T7 (thin lines).

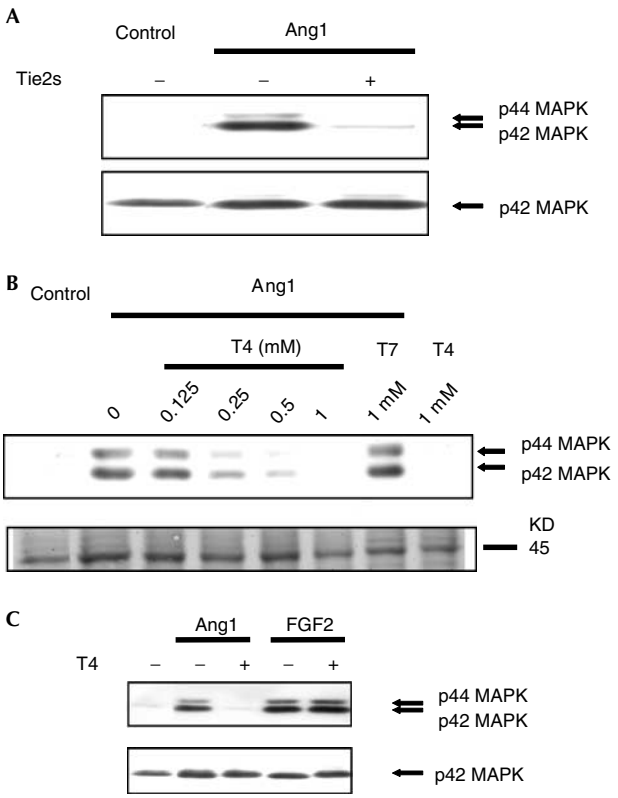

Fig 4 | T4 specifically inhibits Ang1-induced MAPK activation in HUVECs. (**A**) Tie2s blocks p42/p44 MAPK activation induced by Ang1. HUVECs were stimulated with Ang1 with or without Tie2s. (**B**) T4 inhibits the function of Ang1 in a dose-dependent manner. HUVECs were stimulated with Ang1 in the absence (control) or presence of various concentrations of either peptide T4 (0.125, 0.25, 0.5 and 1 mM) or T7 (1 mM). (**C**) Specificity of T4 inhibition. HUVECs were stimulated with Ang1 or FGF2 in the absence (control) or presence of peptide T4 (1 mM). Activation of p42/p44 MAPK was monitored as described in the Materials and methods section. Similar results were obtained in two different experiments.

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migration (Witzenbichler *et al*, 1998). The effect of T4 on the Ang1-induced migration of HUVECs was evaluated. Ang1 induces a >3.5-fold increase in cell migration when compared with the control (Fig 5A,B). This increase was totally inhibited in the presence of peptide T4. On the contrary, T4 did not abolish VEGF-induced migration, demonstrating its specificity.

We next tested its angiogenic activities *in vivo*. We chose the chick chorioallantoic membrane (CAM) assay, which is usually used as an *in vivo* model both to study physiological angiogenesis and to test pro- and anti-angiogenic compounds (Kim *et al*, 2003; Yan *et al*, 2003). The CAM is relevant because Ang1 and Ang2 are expressed within it during angiogenesis (Moyon *et al*, 2001), and the chicken receptor binds both human Ang1 and Ang2 in a similar manner to that of the human receptors (Jones *et al*, 1998). In addition, chicken Ang1 and Ang2 show a high degree of homology to their human counterparts (Jones *et al*, 1998), suggesting that this assay constitutes a pertinent model to test T4 peptide. Because neither Ang1 nor Ang2 alone promote angiogenesis *in vivo* (RT, M-PS, FLN, AE, PE and JP, unpublished observation), we studied the effect of T4 peptide on angiogenesis without addition of exogenous factor. T4 peptide was applied at day 7 ($n=8$; Fig 6), a stage at which strong angiogenesis occurs in the CAM (Kim *et al*, 2003). In seven out of eight CAMs, a striking reduction in vessel density was observed (Fig 6C). In some cases, many zones remained completely avascular (Fig 6D). By contrast, treatment of CAMs with the control T7 peptide ($n=5$; Fig 6B), or PBS vehicle alone ($n=3$; Fig 6A), failed to induce any obvious change in vessel morphology. Soluble Tie2 Fc receptor induces either a weak or no reduction in vessel density (data not shown).

In summary, we showed in this study that peptide T4 (NLLMAAS) inhibited, in a concentration-dependent manner, the

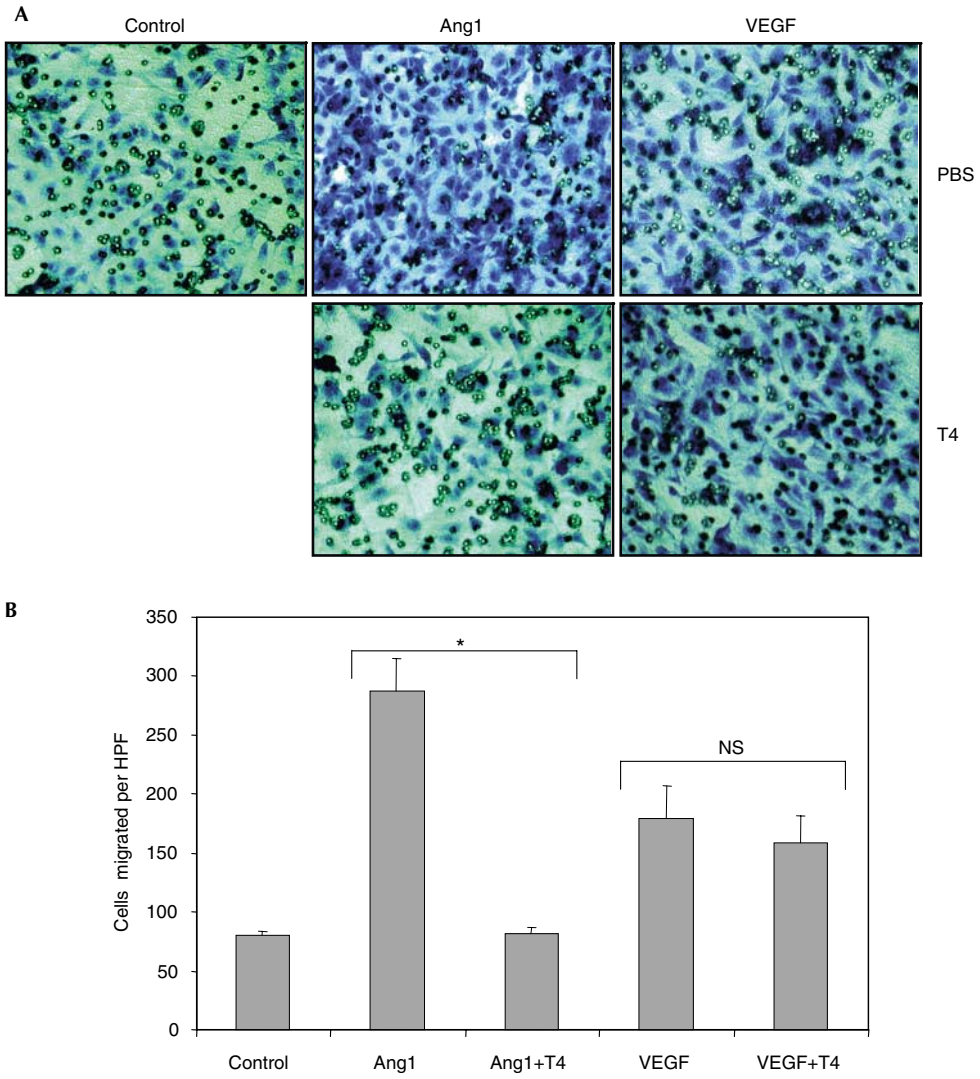

Fig 5 | T4 specifically inhibits Ang1-induced HUVEC migration. HUVECs were allowed to migrate in the presence or absence of Ang1 or VEGF with or without peptide T4 (1 mM). (**A**) Representative picture of each field group. (**B**) Migration score means and standard errors were measured for three fields. Similar results were obtained in two different experiments. *$P<0.05$ versus control. NS, not significant. HUVEC migration was determined as described in the Materials and methods section.

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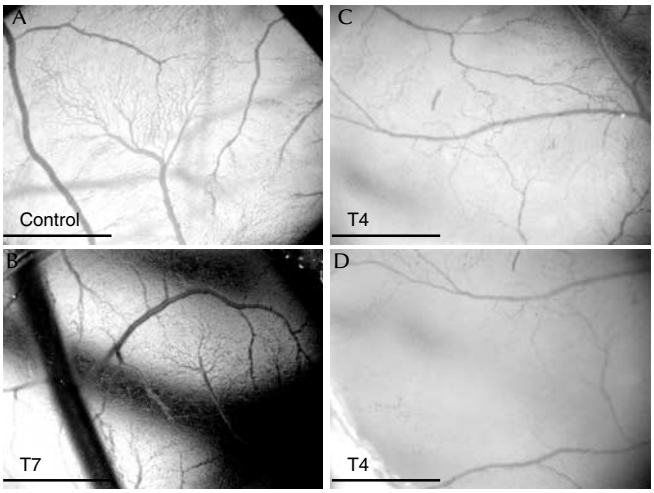

Fig 6 | T4 inhibits CAM angiogenesis *in vivo*. **(A)** A network of blood vessels formed in control PBS-treated embryos. **(B)** No alterations of vessel morphology were observed after treatment with control T7 peptide. **(C,D)** T4 application on the CAM of two different embryos. **(C)** Dramatic reduction of vessel density in the T4-treated region. **(D)** Note the complete loss of capillaries in some regions of the application zone. Scale bar, 1 mm.

binding of Ang1 and Ang2 to immobilized Tie2. It also specifically inhibited Ang1-induced signal transduction and the migration of human endothelial cells. Moreover, we have demonstrated that this peptide can inhibit angiogenesis *in vivo*, in a well-characterized model of angiogenesis—the CAM assay. Although T4 may block both Ang1 and Ang2 binding to Tie2, the resulting effect is an inhibition of angiogenesis.

A comparison of the primary sequence of human Ang1 with that of peptide T4 only revealed an alignment of two residues (LP) with Ang1 at several positions. This may suggest that the binding site of Ang1 on Tie2 is discontinuous and that the selected peptide may contain residues distant in the Ang1 primary sequence, but in close proximity in the folded molecule.

Several studies have shown that interfering with the Tie2 pathway results in murine tumour regression (Lin *et al*, 1997; Siemeister *et al*, 1999). In addition, it has been shown that the proportion of Tie2-positive vessels was significantly higher in breast cancer than in either healthy breast tissue or benign lesions (Stratmann *et al*, 1998, 2001). In highly angiogenic tumours such as glioblastomas, cell-type-specific upregulation of Tie2, Ang1 and Ang2 has been reported (Stratmann *et al*, 1998). Here, we report the first evidence of a peptide (NLLMAAS) interacting with Tie2. This specific inhibitor of the Tie2 pathway could be a good lead compound for the development of therapeutic agents against tumour angiogenesis. The small size of this peptide offers the possibility of designing structurally mimetic nonpeptidic molecules through standard organic synthesis. This could result in the production of inexpensive drugs to be administered orally. Moreover, this bioactive peptide will be useful to dissect the signal transduction mechanisms involving the Tie2 receptor in endothelial cells, which express multiple receptors and may provide a potent tool to inhibit angiogenesis *in vivo*.

MATERIAL AND METHODS

Materials. Human recombinant Ang1, Ang2, biotinylated Ang2, Tie2-Fc, Tie1-Fc and TrkB-Fc were purchased from R&D Systems, whereas recombinant human FGF2 was produced in our laboratory in *Escherichia coli*. $VEGF_{165}$ was obtained from Sigma. The Fc fragment of human IgG was from Jackson Immuno-Research. Peptides were synthesized by Eurogentec.

Cell lines. HUVECs were isolated from umbilical cord veins by collagenase perfusion and were cultivated on gelatinized dishes in SFM medium (Invitrogen) supplemented with 20% fetal calf serum, 100 μg/ml heparin, 20 ng/ml FGF2, 10 ng/ml EGF (Sigma), 50 U/ml penicillin and 50 μg/ml streptomycin. Only cells from passages 2–5 were used for experiments.

ELISA assays. 96-well plates (Maxisorp, Nunc International) were coated with Tie2-Fc or Fc at 4 μg/ml and incubated overnight at 4 °C. Wells were blocked with 0.5% bovine serum albumin. Phage particles (10^{12} particles/ml) were added to each well and incubated for 1 h at 25 °C. Wells were washed 15 times with 0.1% Tween 20 in PBS and the amount of bound phage was detected with peroxidase-conjugated anti-M13 phage antibody (Amersham Pharmacia Biotech). For competition assays, biotinylated Ang2 or Ang1 (200 ng/ml) was added and incubated for 2 h at 25 °C. Bound Ang2 was detected with horseradish peroxidase (HRP)-conjugated streptavidin (Zymed) using 1,2-phenylenediamine dihydrochloride (OPD tablets, DAKO). Bound Ang1 was detected with anti-human Ang1 polyclonal antibody (Santa Cruz Biotechnology) followed by HRP-conjugated anti-goat IgG antibody (DAKO). Tie2s (Tie2-Fc; 30 μg/ml), anti-Tie2 antibody (20 μg/ml; R&D Systems) or peptides were coincubated with Ang1 or Ang2.

Panning the phage library. Biopanning was adapted from the Ph.D.-7 kit standard procedure (New England Biolabs) and has been described previously (Binétruy-Tournaire *et al*, 2000). Tie2-Fc was used to coat microtitre plates at 10 μg/ml.

DNA and amino-acid sequence analysis. DNA sequences were determined by DNA and amino-acid sequence analysis with an A310 sequencer using the ABI PRISM dye terminators (P.E. Biosystems). Alignment between the peptide sequence and the Ang1 or Ang2 primary sequence was determined using the MULTALIGN software.

Surface plasmon resonance (Biacore). Competition assays were performed using a Biacore 2000 instrument. Staphylococcal protein A (Sigma) was covalently immobilized on the carboxymethylated dextran matrix of a CM5 sensor chip (Biacore AB), using the Amine Coupling Kit as described by the manufacturer, to a level of 1,100 RU (resonance units, 1 RU = 1 pg/mm^2). This surface was then used to capture Tie2-Fc by its Fc moiety, to a level of 1,200 RU. Mixtures of Ang1 (0.33 μg/ml) or Ang2 (1 μg/ml) with peptides T4 or T7 (0–1 mM) were then injected at a flow rate of 10 μl/min over the Tie2-Fc/protein A surface for 3 min. Negative controls, obtained by injecting the Ang/peptide mixtures directly onto the protein A surface, were subtracted to determine the specific binding profiles of Ang1 or Ang2 to Tie2 in the presence or absence of peptide. The angiopoietin concentration was chosen to obtain a linear association phase over a time lapse of more than 100 s: the slopes of the different association profiles were measured, and plotted against the concentration of peptide to calculate the inhibition constants (K_i).

MAPK activity. HUVECs were serum starved for 16 h, trypsinized and replated. After 6 h, cells were stimulated for 10 min with Ang1 (300 ng/ml) or FGF2 (100 ng/ml) with or without Tie2s (Tie2-Fc;

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30 μg/ml) or peptide preincubated 1 h before stimulation. Cells were lysed in Laemmli buffer. Proteins were separated on SDS–PAGE (7.5%), and electrophoretically transferred onto polyvinylidone difluoride membrane (Immobilon-P). Membranes were probed with the anti-phospho p42/p44 MAPK monoclonal antibody (Sigma) or anti-p42 MAP kinase antibody (EB14) produced in our laboratory. The immunoreactive bands were visualized with the ECL system (Amersham Pharmacia Biotech).

Cell migration assay. Endothelial cell migration assays were performed using a 24-well chemotaxis chamber (Transwell, Costar), and performed as described (Witzenbichler *et al*, 1998). Cell migration was stimulated with Ang1 (300 ng/ml) or VEGF (10 ng/ml).

***In vivo* CAM assay.** CAM assays were performed as described previously (Le Noble *et al*, 1993). Briefly, fertile normal brown leghorn eggs were incubated for 2 days in a humidified atmosphere at 37 °C. At day 2, a rectangular window was made in the egg shell, the window was covered with scotch tape to prevent dehydration and the eggs were reincubated until day 7. At day 7, a silastic ring was placed on the CAM to allow local drug application. In all, 40 μl of a 10 mM T4 or T7 peptide solution in PBS or PBS vehicle alone was applied in the ring. Eggs were resealed and reincubated for 24 h. *In vivo* pictures were taken using a Leica MZFLIII stereomicroscope equipped with a digital camera (CoolsnapCF, Photometrics) and Metaview analysis software (Universal Imaging Corporation).

Statistical analysis. Significance of differences between groups was tested using a two-tailed Student's *t*-test. Values are represented as mean ± s.d. A *P*-value of less than 0.05 was interpreted as statistically significant.

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